Encapsulation of Fluorescently Labeled RNAs into Surface-Tethered Vesicles for Single-Molecule FRET Studies in TIRF Microscopy

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Encapsulation of fluorescently labelled RNAs into surface-tethered vesicles for single-molecule FRET studies in TIRF microscopy

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ABSTRACT

Imaging fluorescently labelled biomolecules on a single-molecule level is a well-established technique to follow intra- and intermolecular processes in time, usually hidden in the ensemble average. The classical approach comprises surface-immobilization of the molecule of interest, which increases the risk of restricting the natural behaviour due to surface interactions. Encapsulation of such biomolecules into surface-tethered phospholipid vesicles enables to follow one molecule at a time, freely diffusing and without disturbing surface interactions. Further, the encapsulation allows to keep reaction partners (reactants and products) in close proximity and enables higher temperatures otherwise leading to desorption of the direct immobilized biomolecules.

Here, we describe a detailed protocol for the encapsulation of a catalytically active RNA starting from surface passivation over RNA encapsulation to data evaluation of single-molecule FRET experiments in TIRF microscopy. We present an optimized procedure that preserves RNA functionality and applies to investigations of e.g. large ribozymes and RNAs, where direct immobilization is structurally not possible.

Key words: single-molecule FRET (smFRET), lipid vesicle encapsulation, RNA labelling, RNA folding, ribozyme, group II intron

Running head: Encapsulation of RNA for smFRET in TIRFM

1 Introduction

Förster resonance energy transfer (FRET) is a non-radiative transfer of energy between two nearby fluorophores, a donor and an acceptor, overlapping emission and absorption spectra. It is a widely used spectroscopic technique to investigate intra- or intermolecular interactions such as binding events or structural rearrangements of proteins and nucleic acids [1, 2]. FRET is particularly successful on the single-molecule level as it allows to disentangle dynamic processes which obscures short-lived or rare folding intermediates usually averaged out in the molecule ensemble [3–5].

smFRET measurements can be carried out by applying two different methods: confocal or widefield microscopy. In the latter, total internal reflection (TIR) illumination is used to reduce the background signal contribution yielding a high signal-to-background ratio. In TIR fluorescence microscopy (TIRFM), single molecules are usually immobilized on a quartz slide or coverslip with a very low surface density, prior being imaged (Figure 1) [6, 7]. This method allows to follow the folding and reaction kinetics of hundreds of single molecules in parallel over time, only limited by the frame rate (> 100 Hz) of the camera and the limited observation time due to fluorophores photobleaching [8, 9]. The identification of distinct states (model selection) and their interconversion (state transition detection) allows to develop a mechanistic view of the investigated biomolecular system [10].

The immobilization to a functionalized and passivated surface via biotin-streptavidin linkage implies RNA modifications to hybridize a biotin-carrying DNA or PNA oligonucleotide [11, 12] or to covalently link a biotin-moiety to the RNA [13]. Molecule immobilization can cause interactions between the molecule and the surface, which might influence the molecule's fold, and thus leads to misfolding or changes in the associated activity [14]. Further, the surface can alter the photophysical properties of the fluorophores [15]: changes in rotational freedom of the dyes promotes fluorescence fluctuations that yield molecule-unrelated FRET changes [5, 16]. An elegant way to circumvent the disadvantages of a direct surface-immobilization while maintaining prolonged observation periods is to use surface-attached nanoscaled-liposomes which carry the molecule of interest [17, 18]. The vesicles are ~100 nm in diameter and anchored to the surface by a biotin-streptavidine linkage (Figure 1) [17, 19–21]. Thus, the exponential decay of the evanescent excitation field within the vesicle diameter is negligible, and

the excitation probability can be regarded to be nearly constant within the vesicle's diameter. Lipid vesicles membrane permeability becomes maximum near the glass transition T_g of the phospholipids they are composed of [22]. Therefore, the molecules freely diffuse inside the vesicle while the exchange of ions and small molecules is still possible through the vesicles lipid membrane [17]. Further, encapsulating catalytically active biomolecules allows to follow not only folding processes, but also catalytic reactions as both, reactants and products, are trapped inside the vesicle. Recently, it was shown that vesicles have a similar effect on RNA folding as crowding reagents, which chaperone the folding and thus increase RNA fitness [14, 23].

Here, we describe a comprehensive protocol about the encapsulation of fluorescently labelled nucleic acids into phospholipid vesicles. Our model system is a 632 nt-long derivative of the wild-type group IIB intron of *Saccharomyces cerevisiae*, named D135-L14 according to the remaining domains (D) 1, 3, and 5 and two artificial loops L within domains 1 and 4 as labelling platforms [11, 24]. We combine established protocols for surface passivation and vesicle encapsulation and adapt them to retain RNA functionality [17, 19]. Therein, we waive the common freeze-thaw cycles, keep the time of lipid hydration relatively short and adjust the temperature to prevent catalytic activity during the encapsulation process. Applying this protocol enables to successfully encapsulate functional RNAs with a high mono-encapsulation rate and an appropriate vesicle density on the surface. Lastly, we show a comparison of FRET results between surface-immobilized and encapsulated molecules.

2 Materials

All buffers and solutions are prepared with chemicals of at least *purissimum per analysis* quality and purified deionized water. All chemicals were purchased from Sigma-Aldrich and Thermo Fisher Scientific, unless otherwise indicated. To prevent RNA degradation, use sterile-filtered buffer (0.2 μm pore size), gel and salt solutions.

2.1 Lipid vesicles preparation

1. 1 mg DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine): biotin cap PE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (99:1, *see* **Note** 1)
2. 1 mL chloroform
3. extruder and polycarbonate membrane (100 nm, e.g. Avanti Polar Lipids, US)

2.2 Microfluidic chamber and surface passivation

1. quartz slide (7.5 x 2.5 x 0.3 cm)
2. diamond driller (1 mm)
3. glass coverslip (24 x 24 mm, type #1: thickness 0.13 – 0.16 mm, to be used with a high numerical aperture (NA) water-immersion objective (NA 1.2, magnification 60x, working distance >0.27 mm)
4. 80 mg Methoxy-Poly(Ethylene Glycol)-Succinimidyl Valerate (MW 5000 g/mol, mPEG-SVA-5000)
5. 2 mg Biotin-PEG-SVA-5000 (*see* **Note** 1)
6. imaging spacer (thickness 0.12 mm, e.g. SecureSeal^{TM} from Grace Bio-Labs, US)
7. plasma cleaner (e.g. Diener electronics, Germany)

2.3 Reagents for RNA labelling, encapsulation and imaging

1. Fluorescently labelled (and biotinylated) DNA oligonucleotides were purchased from IBA (Göttingen, Germany), and PNA oligonucleotides were synthesized as described elsewhere [12]. Any molecule carrying a dye, always needs to be protected from light.
2. 3 mg/mL Trolox
3. 20 µL/mL streptavidin in 10 mM phosphate buffered saline, pH 7.4 (KOH)

4. 2170 U/mL catalase
5. 165 U/mL glucose oxidase
6. T50 buffer: 50 mM NaCl, 50 mM TRIS, pH 7.5 (NaOH) [25]
7. OSS solution (100x): 22 U catalase, 1.7 U glucose oxidase, T50 buffer (see **Note 2**) [26, 27]
8. SB - Standard buffer (5x) to be adapted to the optimized folding and activity conditions of the investigated RNA: 2.5 M KCl, 0.4 M MOPS, pH 6.9 (KOH), sterile filtered, store under darkness at 4 °C [28]
9. AB – Anti-blinking buffer (10 mL): 2 mL of 5x SB, 0.1 M MgCl2, 3 mg/mL Trolox, pH 6.9 (KOH) (see **Note 3**) [29, 30]
10. SAB – Sugar-anti-blinking buffer: 1 mL of AB, 1% w/v D-glucose (see **Note 4**)
11. IB - Imaging buffer: 495 µL SAB, 5 µL 100x OSS (see **Note 4**)

3 Methods

3.1 Lipid cake preparation

1. Dissolve 0.1 mg of phospholipids (99:1 DMPC:biotin-cap PE) in 100 µL chloroform [17].
2. Prepare a thin phospholipid layer deposited on the round bottom of a 1.5 mL reaction tube, called lipid cake, by evaporating the chloroform under nitrogen flow for at least 2 h [17, 19]. Remove remaining traces of solvent under vacuum overnight (*see* **Note 1**).

3.2 Site-specific labelling of long RNAs

Prior to labelling the RNA of interest, here the D135-L14 ribozyme construct, is prepared by *in vitro* transcription with home-made T7 RNA polymerase and purified by means of polyacrylamide gel (5%) electrophoresis (PAGE) under denaturing conditions [31].

1. Incubate 1 µM of purified RNA in SB for 1 min at 90 °C.

2. Decrease the temperature to 42 °C for 3 min allowing secondary structures to form [12].

3. Add fluorescently labelled DNA [11] or PNA oligonucleotides [12] carrying each a sulfonated fluorophore, the donor (sCy3) and acceptor (sCy5), which are complementary to the respective labelling sites, in a 1:1 ratio (*see* **Note 5**) to the RNA for hybridization illustrated in Figure 2 (*see* **Note 6**).

4. The folding of the RNA is induced by adding 100 mM $MgCl_2$. Please note, that the specific folding conditions need to be adapted for any RNA under investigation. The ribozymes activity, here the cleavage reaction of D135-L14, can be followed by incubating the RNA at 42 °C or stalled at 22 °C to follow the folding only.

Site-specific labelling can also be achieved by DNA-helper-strand-guided post-transcriptional base modification and subsequent bi-orthogonal RNA labelling as presented recently [32].

3.3 RNA encapsulation into phospholipid vesicles

1. Prepare a 200 nM solution of fluorescently labelled RNA with a final volume of 100 µL.

2. Hydrate the prepared lipid cake with the 100 µL RNA solution; incubate both stirring for 5 min, 30 °C, *i.e.*, above DMPC glass transition (T_g = 23°C), at 1400 rpm, followed by 20 min, 30 °C at 700 rpm (*see* **Note** 7) [19].

3. Meanwhile, assemble the extruder according to the instructions of the manufactory. The two filters left and right from the membrane have to pre-equilibrate in dH_2O.

4. Equilibrate the polycarbonate membrane with 100 nm-diameter pores inside the extruder with 1 mL AB. Take up 1 mL AB with one syringe, mount the syringe accordingly and push the buffer twice through the extruder.

5. Place the extruder on a heating block and let it warm up to 30 °C (*see* **Note 8**).

6. Centrifuge the lipid-RNA solution for 2 min at 12'000 rpm to remove tiny vesicles (Figure 3).

7. Dilute the suspension with 500 µL of AB to a final RNA concentration of 33.3 nM by gently re-suspending the solution (*see* **Note 9**).

8. Extrude the cloudy suspension at 30 °C 35 times through the polycarbonate membrane, which determines the final size of the vesicles (here 100 nm). The obtained RNA-vesicle sample is ready for the immobilization step e.g. in a microfluidic chamber.

3.4 Vesicle and dye characterization

Vesicle diameter (100 nm) has been chosen in such a way, that the RNA of interest (here 16 nm) can diffuse freely, the influence of the confined environment on the RNA is reduced as much as possible [14, 23], and intensity fluctuations due to the exponential decay $\sim \exp(-z/d)$ of the evanescent field along the optical axis z are negligible (Figure 3). The latter is realized by adapting the penetration depth $d = \lambda/4\pi\sqrt{n_{\mathrm{quartz}}^2 \sin^2\theta - n_{\mathrm{H_2O}}^2)} \leq 70$ nm of the evanescent field which can be varied by changing the incidence angle θ (Figure 3); the refractive indices $n_{\mathrm{quartz}} = 1.46$ and $n_{\mathrm{H2O}} = 1.33$ as well as the wavelength of the incident laser beam in vacuum $\lambda = 532$ nm are determined by the experimental setup and cannot be changed.

It has been reported, that the fluorophore-labels of the encapsulated biomolecules interact with the phospholipid membrane, changing the fluorophores photophysics, thus, yielding unreliable FRET traces [34]. We therefore encapsulated carbocyanine dye labelled DNA oligonucleotides yielding constant single-molecule fluorescence intensity time traces (no blinking) and no changes in the fluorescence lifetime and dynamic fluorescence anisotropy (no intercalation into the membrane) proving that the dye – membrane interaction is negligible. Further, we recommend the use of sulfonated-carbocyanines to reduce the interaction propensity of the dye with the negatively charged RNA backbone. In addition, carbocyanines show changes in their quantum yield upon interacting with the RNA due to changes of their cis-trans isomerization propensity, known as RNA-induces fluorescence enhancement (RIFE) [33]. Rigid carbocyanines, such as Cy3B, cannot undergo a cis-trans isomerization and are thus RIFE-free.

3.5 Microfluidic chamber preparation including surface passivation for prism-based TIRFM

Prepare the self-made microfluidic chamber prior to the smFRET experiment [35]:

1. Drill two holes per chamber into the quartz slide according to the chamber design e.g. provided by the imaging spacer for flushing the required solutions, using a diamond driller.
2. Rinse and sonicate the quartz slide (and the coverslip) for 20 min at r.t. successively with dH_2O, technical acetone and dH_2O.
3. Clean and activate the surface of the quartz slide and the coverslip with a plasma cleaner for 30 min and low-pressure oxygen-atmosphere according to standard protocols.
4. Both surfaces of the chamber are aminosilanized, followed by overnight PEGylation according to standard protocols [36]. Incubate the coverslip and quartz slide with 30 µL a solution containing 99 mg mPEG-SVA-5000 and 1 mg Biotin-PEG-SVA-5000 dissolved in 1 mL of 0.1 M sodium carbonate buffer. (*see* **Note 10**)
5. After successively washing with dH_2O and drying with nitrogen gas stream, join the drilled quartz slide and the coverslip with a double-sided adhesive imaging spacer thus, building the microfluidic sample chamber. (*see* **Note 11**)
6. The PEGylated chambers can be stored in falcon tubes under nitrogen atmosphere at −20 °C and for one month without observing quality loss.

3.6 smFRET experiment

1. Flush the chamber once with 200 µL of T50 buffer before coating the surface with 100 µL streptavidin (20 µL/mL) for 10 min at r.t.
2. Remove unbound streptavidin by a washing step with 100 µL AB.
3. Immobilize 100 µL of the lipid vesicle suspension and incubate for 10 min at r.t. In case of a direct surface immobilization (Figure 4), the vesicle suspension is replaced by the sample

solution containing the biotin-functionalized and fluorescently labelled RNA of interest with a pM concentration.

4. Meanwhile, prepare fresh IB containing SAB, the oxygen scavenger system (OSS) and Trolox to prevent early photobleaching of the fluorophores; flush the chamber finally with 200 µL IB and incubate for another 5 min prior imaging.

5. Seal the drilled holes in the quartz slide to prevent evaporation and mount the chamber onto the water-immersion objective (*see* **Note 12**).

6. Use a drop of immersion oil before placing the quartz TIRF prism onto the quartz slide of the chamber (*see* **Note 13**).

7. The temperature of the objective and the sample holder can be controlled by a temperature controller (*see* **Note 14**).

3.7 Single molecule video (SMV) processing and data analysis

SMV processing for TIRF-based smFRET experiments is presented elsewhere [38]. However, a number of mandatory steps is described herein. We recommend to perform data analysis of smFRET movies by using our Multifunctional Analysis Software for heterogeneous FRET data (MASH-FRET) (https://github.com/RNA-FRETools/MASH-FRET.git) [10, 39] or iSMS [40], both Matlab-based home-written software packages:

1. Single molecules are localized on the SMVs and fluorophore emission intensity trajectories in imaging counts per frame (IC/frame), but usually re-calculated in counts per second (a.u./s) are created as described elsewhere [39].

2. Trajectories are individually corrected from the background signal by using the "median" method [41]. Photon emission detected in the Cy5 channel is reduced by 7 % of Cy3 photon emission (bleed-through) and 2 % of Cy5 photon emission upon 638 nm excitation (direct excitation) summarized elsewhere [39, 42].

3. Single encapsulation is ensured by selecting trajectories displaying Cy3 and Cy5 emission. Single dye emission level is estimated from single bleaching step trajectories upon respective direct excitations. The fluorescence intensity trajectories selected for analysis fulfil the following criteria:

- $I_{\mathrm{Cy5\,em}}^{\mathrm{Cy5\,ex}}(t)$ must appear stable and correspond to single Cy5 emission level along the observation time (no defocusing, blinking, photobleaching nor inferring neighbouring molecules),

- total photon emission upon Cy3 excitation ($I_{\mathrm{tot\,em}}^{\mathrm{Cy3\,ex}}(t) = I_{\mathrm{Cy3\,em}}^{\mathrm{Cy3\,ex}}(t) + I_{\mathrm{Cy5\,em}}^{\mathrm{Cy3\,ex}}(t)$) must appear stable and should correspond to a single Cy3 emission level without energy transfer along the observation time,

4. The trajectories for apparent energy transfer *FRET* (*t*) and stoichiometry *S*(*t*) is calculated using the respective equations:

$$FRET_{\mathrm{PR}}(t) = \frac{I_{532,\mathrm{Cy5em}}(t)}{I_{532,\mathrm{Cy5em}}(t) + I_{532,\mathrm{Cy3em}}(t)} \tag{1}$$

$$S(t) = \frac{I_{532,\mathrm{Cy5em}}(t) + I_{532,\mathrm{Cy3em}}(t)}{I_{532,\mathrm{Cy5em}}(t) + I_{532,\mathrm{Cy3em}}(t) + I_{638,\mathrm{Cy5em}}(t)} \tag{2}$$

$FRET_{\mathrm{PR}}$ values can be corrected for possible variations in quantum yield and photon detection efficiency, called γ-correction [43, 44], yielding true *FRET* values that can be converted into absolute distances between the two labelling position L1 and L4 [42, 45]. The time traces selected for analysis exhibit both dyes within one vesicle, resulting in an average stoichiometry $S \approx 0.5$.

5. Multi-or mono-encapsulation can be distinguished by considering the fluorescent intensities, the stoichiometry and single-step photobleaching (Figure 5).

6. Selected single molecules can be classified into "dynamic" and "static" according to the presence or the absence of *FRET* state transitions, *i.e.*, detectable by eye or using model selection and state transition detection methods discussed elsewhere [10].

7. Contributions of all selected trajectories are summed into a 0.01 *FRET*-binned histogram to obtain the ensemble FRET probability distribution (Figure 4).

8. A stable or transient state in the histogram is modelled with a Gaussian distribution, centred on the state's *FRET* value. The presumed relative state populations are accurately characterized by fitting a mixture of Gaussian functions to the histograms (Figure 4). The Gaussian mixture model (GMM) inferred via BIC-selection is obtained by using an expectation-maximization (E-M) method for likelihood maximization [45].

9. In order to correct for differences in the detection efficiency of both detection channels, which enables the comparison of FRET results between different microscope setups, as well as to correct for differences of fluorophore quantum yields, which allows to use FRET as a distance measure, gamma-correction is carried out. We compared the gamma factor distribution of surface immobilized with encapsulated RNA molecules fluorescently labelled with Cy3/5 and found almost no difference besides of an increasing number of outliers and thus a broadening of the distribution for encapsulated molecules (Figure 4).

4 Notes

1. Biotin is degrading over time; the expiration date of the lipids should not exceed one year. Lipid cakes were stored at −20 °C for a maximum duration of one month and no loss of quality was observed.

2. T50 buffer is used for the respective OSS, but can be replaced by SB.

3. [Mg2+] to be adapted to the optimized folding and activity conditions of the investigated RNA [11, 28].

4. Both, the IB and the SAB, should not be used longer than one week.

5. The ratio of RNA to fluorescently labelled DNA or PNA oligonucleotide is crucial for mono-encapsulation. If the label is in excess over the RNA, the probability of encapsulating DNA or PNA oligonucleotides only is very high.

6. High temperatures can destroy the fluorescent dyes; therefore, the labels should be added after the denaturation step at 90 °C. To increases the overall labelling efficiency, the design of dye-carrying DNA or PNA oligonucleotides is rationalized as follows:

    - The RNA labelling site is unique within the RNA sequence and reports on dynamics important for folding and function.

    - The size of the DNA oligonucleotides should be as short as possible to reduce necessary modifications of the RNA sequence or - if binding to the natural sequence does not influence the RNAs functionality – to reduce the influence on the RNA folding. In both cases, the GC content should be chosen is such a way to yield ideally a melting temperature above 60°C of the hybrid. In contrast to DNA, PNA are without charge and thus have a higher binding affinity towards RNA. For that reason, PNAs can be kept much shorter (down to 8 – 10 nt) and retain similar hybrid stability [12].

    - The labelling efficiency via hybridizing complementary oligonucleotides depends on the respective RNA and on the length and type (PNA or DNA) of the oligonucleotides [12]. Labelled and unlabelled molecules were left unseparated prior encapsulation. Molecular sorting is further performed by means of alternating laser excitation (ALEX), which enables to identify single labelled species [43, 46].

7. The ratio of RNA to lipids is very important to obtain mono-encapsulated RNAs.

8. We are interested to follow the functional RNA before, during and after its catalytic step. Therefore, it is important to prevent the catalytic step (here the cleavage reaction of D135 with its substrate 17/7 *in trans*) during the encapsulation. This is achieved by keeping the temperature relatively low at 30°C and adding magnesium(II) ions as late as possible; *in vitro* cleavage does not take place at temperatures below 30 °C and at low magnesium(II) concentrations.

9. The volume of the lipid-RNA mixture depends on the size of the syringes and the dead volume, here we use two 1 mL syringes. The RNA concentration is crucial to ensure an appropriate density of fluorescently labelled RNAs.

10. A mixture of PEG/biotinylated PEG (99:1) is used for vesicles immobilization. The well-known surface passivation and immobilization via BSA/biotin-BSA cannot be used here as the vesicles loose surface contact and roll along the surface, in particular, upon rising the temperature above 40°C.

11. The thickness of the imaging spacer and the coverslip must be smaller than the working distance of the used WI objective.

12. Sealing the quartz slide holes is especially important at higher temperatures to prevent evaporation of the solution inside the chamber.

13. Use immersion oil for quartz only ($n_{\text{quartz, 532 nm}} = 1.46$).

14. Wait 10 min until the system including the microscope objective, immersion water, immersion oil, and the sample chamber itself equilibrate to the set temperature. Otherwise, you will lose focus meanwhile the acquisition time. Using both, an objective and a sample holder heating device, prevent for a temperature gradient within the sample chamber.

Application notes:

- The TIRFM setup design for single-molecule FRET measurements is described elsewhere [35, 47, 48].

- Encapsulation of NA [14, 17, 20] and proteins [18, 49] is used to investigate their binding interactions, folding and chemical reactions.

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Figure Legend

Figure 1. To scale scheme of both immobilization techniques: Classical surface immobilization of a large RNA (left) versus vesicle encapsulation (right). The group II intron ribozyme construct D135-L14 is either directly tethered to the biotin-PEG-passivated quartz surface via a biotinylated DNA oligonucleotide hybridizing to the extended 3'-end of the RNA (left) or freely diffusing in a phospholipid vesicles, which itself is tethered in the respective way (right).

Figure 2. Labelling scheme of D135-L14, a derivative of the wild-type group II intron *Sc*.ai5γ. The RNA is labelled via artificially introduced loops in domain D1 and D4 (L1, L4). Complementary DNA oligonucleotides (black) or PNAs (blue) carrying a fluorescent dye, here sCy3 (donor) and sCy5 (acceptor) can hybridize to these labelling platforms. The corresponding melting temperatures (T_m) of the hybrids were determined experimentally. D135-L14 can be directly immobilized on a streptavidin-coated surface via the biotinylated DNA oligonucleotide complementary to the 3' elongation.

Figure 3. Vesicle properties. (a) Exponentially decaying evanescent field in TIRFM in comparison to the size of the lipid vesicle. Decay of the excitation probability over the vesicle diameter is less pronounced for smaller vesicles; excitation probability fluctuations within the integration time of the camera average out due to RNA diffusion within the vesicle. (b) Size distribution of vesicles measured by means of dynamic light scattering (DLS) after using the extruder, but without centrifugation necessary to remove too small vesicles prior to extrusion. (c) Fluorophore characterization. Fluorescence lifetime (top) and dynamic anisotropy (bottom) measurements of carbocyanines, the donor sCy3 (left) and the acceptor sCy5 (right), free in solution, covalently attached to DNA-oligonucleotides, DNA-oligonucleotides hybridized to D135-L14 and the labelled D135-L14 encapsulated into lipid vesicles. The lifetime of both carbocyanines increases upon decreasing the cis-trans isomerization propensity in presence of DNA-oligonucleotides and RNA, respectively [33]. The decreased decay time of the dynamic anisotropy supports this finding. The lifetime and dynamic anisotropy of the labelled RNA free in solution and encapsulated are similar, thus, the encapsulation does not alter the characteristics of the fluorophores and a possible interaction of the fluorophores and/or RNA with the lipid membrane can be

neglected. Upon encapsulation of the labelled RNA the scatter background increases drastically, limiting the observation window to a few nanoseconds.

Figure 4. Comparison of (top) the direct surface-immobilized and (bottom) encapsulated D135-L14 ribozyme with a (right) typical FRET trajectory and (middle) the ensemble FRET probability histograms of *n* molecules. Although the FRET histograms are almost identical, which demonstrates the negligible effects of the encapsulation on the RNA folding, the population of higher FRET states increases upon encapsulation ($\Delta F + \Delta N = 3\%$). Further, state transitions to higher FRET states appear more frequently upon encapsulation. These results are in line with previous observations of the ribozyme in a crowded, and thus confined, environment [23]. Error bars were obtained via bootstrapping [37]. Further, the γ-factor for individual molecules was determined according to the protocol proposed by McCann et al [45]. (very right) The characterization of the γ-factor distribution is summarized as box plot centred around the mean value with one standard deviation box height. The median value of the distribution is drawn as horizontal line (0.59 for surface immobilized and 0.61 for encapsulated molecules).

Figure 5. Examples of smFRET trajectories (30 frames/s) using millisecond ALEX in prism-based TIRFM showing (left) double- and (right) mono- encapsulation. (top) The fluorescence intensities (red, green and yellow) as well as the calculated FRET efficiency (grey) and stoichiometry (blue) are shown together with their respective probability histograms. (left) The intensity trajectory shows a bleaching step of the acceptor dye, Cy5 (red), after 175 s (arrow), indicating a double-encapsulation. (right) The intensity trajectory shows a bleaching step of Cy5 too (arrow). In contrast, the fluorescence of Cy5 drops to zero, while at the same time the stoichiometry goes up from 0.5 to 1, clearly indicating a mono-encapsulation.